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Retinoid Status Controls the Appearance of Reserve Cells and Keratin Expression in Mouse Cervical Epithelium

Nadine Darwiche, Giulia Celli, Linda Sly,[1] Francesca Lancillotti,[2] and Luigi M. De Luca[3]

Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, NIH, Bethesda, Maryland 20892

ABSTRACT

We describe an animal model to induce the histogenesis of squamous metaplasia of the cervical columnar epithelium, a condition usually preceding cervical neoplasia. This model is based on dietary retinoid depletion in female mice. Control sibling mice fed the same diet but with all-*trans*-retinoic acid (at 3 μg/g diet) showed the normal endocervical epithelial and glandular columnar morphology, typical of a simple epithelium without subcolumnar reserve cells. The stratified squamous ectocervical epithelium of these mice fed all-*trans* retinoic acid showed intense immunohistochemical staining in basal and suprabasal cells with monospecific antibodies against keratins K5, K14, K6, K13, and, suprabasally, with antibodies specific for K1 and K10. At the squamocolumnar junction, the adjacent columnar epithelium (termed "suprajunctional") did not show staining for K5, K14, K6, K13, K1, and K10 but specifically stained for keratin K8, typical of simple epithelia and absent from the adjacent ectocervical squamous stratified lining (termed "subjunctional"), in striking contrast. Sections of the squamocolumnar junction from mice kept on the vitamin A-deficient diet for 10 weeks showed suprajunctional isolated patches of reserve cells, proximal and distal to the junction. These cells were detected prior to any symptoms of vitamin A deficiency, such as loss of body weight or respiratory discomfort. The subcolumnar reserve cells induced by vitamin A deficiency displayed positive staining for K5 and K14. As deficiency became severe, the reserve cells occupied the entirety of the suprajunctional basement membrane. This epithelium eventually became stratified and squamous metaplastic, the squamocolumnar junction was no longer discernible, and the entire endocervical epithelium and the endometrial glands lost K8 positivity, while acquiring K5, K14, K6, K13, K1, and K10 keratins typical of the ectocervix under normal conditions of vitamin A nutriture. Vitamin A deficiency also altered keratin expression and localization in squamous subjunctional epithelium. *In situ* hybridization studies for K1 and K5 mRNA showed their major site of expression at the basal (K5) and immediately suprabasal (K1) cell layers. The localization of both K5 and K1 proteins in these same cell layers, and above, is consistent with transcriptional regulation of these keratins. Early vitamin A deficiency caused the appearance of single subcolumnar reserve cells expressing K5 mRNA. After these cells grew into a squamous focus, K1 mRNA became expressed suprabasally. We conclude that retinoid status plays a key role in maintaining differentiative characteristics of the cervical and glandular epithelia and, as such, may be a modulating factor in the development of cervical cancer.

INTRODUCTION

Squamous metaplasia marks the replacement of simple, transitional, or pseudostratified epithelial linings with a squamous stratified type of epithelium (1). Common targets of squamous metaplasia are the site of neoplastic disease and include the bronchus (2, 3), trachea (4), stomach (5, 6), urinary bladder (7), and the cervical columnar epithelium (8, 9). The cervix uteri is lined by a stratified squamous epithelium and a simple columnar epithelium, which join abruptly at the squamocolumnar junction or transformation zone (Fig. 1). This squamocolumnar junction and its two adjacent epithelial phenotypes can be located either in the endocervical canal or in the ectocervix, depending on age, hormonal, and other conditions. Therefore for ease of presentation we will refer to cells on the endocervical side of the junction as "suprajunctional" and to cells on the vaginal side as "subjunctional." Usually suprajunctional cells are columnar, whereas subjunctional cells are squamous (see Fig. 1). We aim to define signal molecules involved in maintaining the morphological and functional characteristics of the squamocolumnar junction in an effort to identify preventive mechanisms against the development of cervical neoplasia.

Retinoids are essential for maintenance of normal epithelial morphology and function (reviewed in Ref. 10) in the adult and for the control of morphogenesis in the embryo (11, 12).

In addition steroid hormones are known to be involved in the maintenance of normal morphology and function of the cervical and vaginal epithelia and an antagonistic action between estrogen\androgen and retinoic acid has been characterized in various tissues, including the tracheal, prostate, and cervical epithelia (13, 14). Usually estrogen induces the keratinizing phenotype during the estrous phase of the menstrual cycle in squamous stratified nonkeratinizing epithelia, and progesterone and RA[4] antagonize this response (15, 16). Also vitamin A deficiency causes squamous metaplasia and keratinization of the epithelium lining the uterine cavity and glands in the rat (17) and guinea pig (18).

Since both steroids and retinoids exert their action via their nuclear receptors (reviewed in Ref. 19), the maintenance of the normal morphology and function of the cervical epithelium may well depend on the topology of the specific expression of these transcriptional regulators, their heterodimeric interactions and the availability of their ligands. Whereas endocrine mechanisms regulate the synthesis and availability of steroid hormones, nutritional intake and tissue homeostasis are responsible for the presence of retinoids within target tissues. Although both of these mechanisms are important in maintaining normal epithelial functions, nutritional factors have received little attention.

Therefore we resorted to purified diets to control retinoid intake and induce the phenotype of squamous metaplasia in the cervical columnar epithelium and glands of the mouse uterus. This nutritional regimen, the use of specific keratin antibodies, and the technique of *in situ* hybridization have enabled us to identify the gradual appearance of reserve cells in focal and discontinuous distribution patterns, suprajunctionally within the cervical epithelium and endometrial glands. These reserve cells eventually grow into squamous metaplastic foci, the preneoplastic lesion of squamous cell carcinoma (20, 21). We describe here a mouse model that permits the study of the histogenesis of squamous metaplasia of the cervix under controlled conditions of dietary intake of RA and the characterization of keratin expression during this process.

MATERIALS AND METHODS

Preparation of Vitamin A-deficient Mice. Female BALB/c and nude mice and their mothers were placed on the vitamin A-deficient test diet (TD 85239; Teklad, Madison, WI) at birth of the experimental animals following a protocol developed for SENCAR mice (22). They were weaned at week 3 of age and

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[1] Present address: Biocon, 649 A Lofstrand Lane, Rockville MD 20850-1360.

[2] Present address: Instituto Superiore di Sanità, Laboratorio di Virologia, Viale Regina Elena 299, 00161 Rome, Italy.

[3] To whom requests for reprints should be addressed, at Building 37, Room 3A-17, NIH, Bethesda, MD 20892.

[4] The abbreviations used are: RA, all-*trans*-retinoic acid; K, keratins; HPV, human papilloma virus.

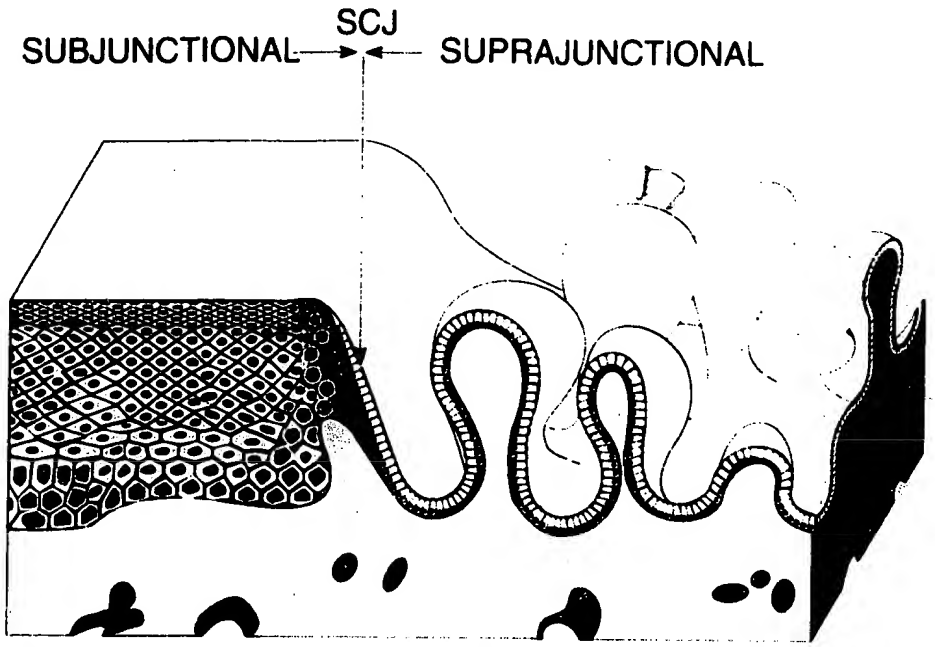

Fig. 1. Schematic representation of squamocolumnar junction (*SCJ*) with stratified squamous (subjunctional) epithelium and simple columnar (suprajunctional) epithelium. Reprinted with permission from Ref. 34.

maintained on the deficient diet for periods of time such that mild (10 weeks for nude mice and 14 weeks for BALB/c mice) and severe (14 weeks for nude mice and 20 weeks for BALB/c mice) vitamin A deficiency would ensue, as monitored by loss of body weight and liver retinylpalmitate levels.[5] Control mice were fed the same diet but with the addition of RA at physiological levels (3 μg/g) (22).

Immunohistochemistry. Mice were sacrificed at specified times and the uterus and vagina were removed and fixed quickly in 70% ethanol at 4°C. Paraffin-embedded sections (5 μm) were usually prepared so as to include the subjunctional and the suprajunctional epithelia for immunohistochemical staining. Sections were deparaffinized in xylene and hydrated with successive bathing in 100, 95, 80, and 70% ethanol. They were then incubated with the primary antibodies diluted in 12% bovine serum albumin in phosphate-buffered saline for 2 h at room temperature. Affinity-purified rabbit antiserum (23) specific for keratin K6, K10, K5, K13, or K14 and guinea pig anti-peptide antiserum for K1 was used. Antisera against K1, K6, and K10 were generated against carboxyl-terminal peptides of published sequences (24–26). K14 anti-peptide antiserum (27) and K8-specific antiserum (28) were generated as described. The sections were exposed to biotinylated goat anti-rabbit secondary antibodies or biotinylated goat anti-rat for K8 detection and the Vectastatin ABC kit was used (Vector, Burlingame, CA). Peroxidase staining was performed using Histomark Streptavidin-HRP Systems obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). The procedure used follows the original method as described by Gill *et al.* (29). The working solution was identical to that described by Graham and Karnovsky (30). Sections were also stained with contrast green (KPL, Bethesda, MD). After washing, the sections were mounted in Accu-mount and viewed under light microscopy.

***In Situ* Hybridization.** Cervical frozen sections (5 μm) were mounted on gelatin-coated slides and stored at −70°C until used. *In situ* hybridization was performed according to the protocol of Young (31). The hybridization buffer contained 2×10^6 dpm [^{35}S]CTP/100 μl of final buffer (20 mM Tris-HCl, pH 7.4–1 mM EDTA, pH 8–300 mM NaCl-50% formamide-10% dextran sulfate-1× Denhardt's solution-100 μg/ml salmon sperm DNA-250 μg/ml yeast total RNA-250 μg/ml yeast tRNA-1% sodium thiosulfate-1% sodium dodecyl sulfate-100 mM dithiothreitol). Sections were incubated at 50°C overnight in a humid chamber. Following the procedure as detailed in Ref. 31, the sections were dipped into LM-1 emulsion (Amersham), and exposed in the dark at 4°C for 2 weeks. The slides were developed in Dektol developer and Kodak fixer. The sections were then stained with hematoxylin and eosin.

Preparation of Riboprobes. RNA transcripts were synthesized from mouse complementary DNA fragments of the K1 and K5 3′-non-coding region (400 and 350 base pairs, respectively, cloned in pGem 3) (Promega), from both strands using T7 and SP6 RNA polymerase and [^{35}S]CTP (32, 33).

[5] N. Darwiche *et al.*, manuscript in preparation.

RESULTS

Induction of Vitamin A Deficiency. Fig. 1 (adapted with permission from Ref. 34) shows a schematic drawing of the cervical area surrounding the squamocolumnar junction. It emphasizes the sharp switch from the squamous stratified morphology of the subjunctional epithelium (usually in the ectocervix) to the suprajunctional simple columnar phenotype, usually found in the endocervical canal and glands.

We monitored the induction of vitamin A deficiency by following body weight, liver retinyl palmitate (results not shown), as well as epithelial morphology of the cervix in nude and BALB/c mice. In nude mice vitamin A deficiency could be induced in shorter times than in BALB/c mice (results not shown). Fig. 2 presents a panoramic view of the morphological changes, as monitored by hematoxylin and eosin staining, taking place in the area of the squamocolumnar junction (Fig. 2, *A* and *B*) during mild (Fig. 2, *C* and *D*) and severe vitamin A deficiency (Fig. 2, *E* and *F*). Under normal conditions of retinoid nutriture (RA at 3 μg/g diet (Fig. 2, *A* and *B*) the subjunctional epithelium retains the normal stratified squamous morphology which becomes keratinized in the estrous phase of the menstrual cycle (35) or under conditions of extreme vitamin A deficiency (Fig. 2, *E* and *F*). Feeding a vitamin A-deficient diet for 10 weeks in nude mice and for 14 weeks in BALB/c mice causes the suprajunctional appearance of foci of squamous metaplasia which eventually populate the entire columnar epithelium, giving it a squamous stratified appearance (Fig. 2*F*). This epithelial change also occurs in endocervical glands (Fig. 2*F*).

Expression of Keratins 5 and 8. Next we used keratin-specific antibodies to monitor the formation of squamous lesions. Antibody to keratin K5 specifically stained the subjunctional stratified epithelium at the basal and suprabasal level (Fig. 3*A*), whereas antibody to K8 specifically stained the suprajunctional simple columnar epithelium of the endocervical canal and endometrial glands (Fig. 3*B*). The condition of vitamin A deficiency at an early stage (Fig. 3*C*) caused distinct staining of squamous foci in a dispersed pattern, proximal as well as

Fig. 2. Sections, (5 μm) of cervices from BALB/c mice maintained on different retinoid diets. In this and subsequent figures the vaginal side ("subjunctional") is on the left and the endocervical side ("suprajunctional") is on the right. RA+ (3 μg/g) diet: *A*, × 17; *B*, × 70. Mild vitamin A deficiency: *C*, × 17; *D*, × 70. Severe vitamin A deficiency: *E*, × 17; *F*, × 70. H & E.

distal to the junction. Severe vitamin A deficiency, as shown in Fig. 3*E*, causes a near complete replacement of the columnar suprajunctional epithelium and glands by a stratified keratinized layer of cells (Fig. 3*E*). It should be emphasized that the acquisition of K5 positivity coincided with the loss of K8 expression (Fig. 4, *E* and *F*). Vitamin A deficiency at either stage did not seem to influence the subjunctional expression and localization of K5 compared to control epithelium.

Expression of Keratins K14 and K1. Fig. 4 shows the immunohistochemical staining with antibodies to keratins K14 and K1. K14 was found to colocalize with K5 (Fig. 4, *A*, *C*, and *E*) and therefore

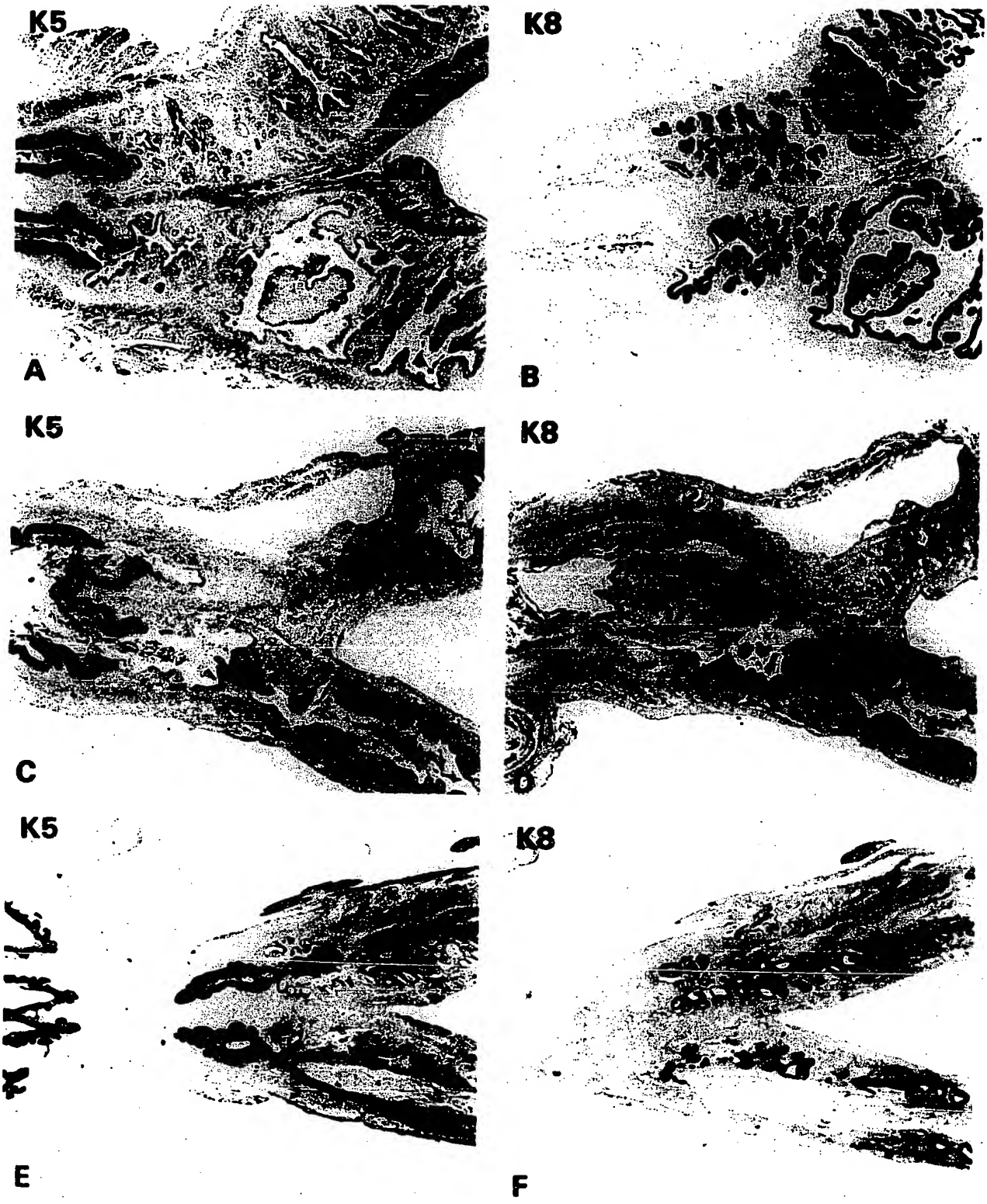

Fig. 3. Immunohistochemistry of keratins K5 (*A, C, E*) and K8 (*B, D, F*) in cervical sections of BALB/c mice. *A, B*, RA+ diet; *C, D*, mild vitamin A deficiency; *E, F*, severe vitamin A deficiency. × 17.

is equally modulated by vitamin A deficiency. Antibody to keratin K1 stained the stratified subjunctional epithelium at the suprabasal level (Fig. 4*B*), without staining in the suprajunctional epithelium. The condition of vitamin A deficiency at an early stage (Fig. 4*D*) caused distinct staining of suprajunctional squamous foci in a dispersed pattern, proximal as well as distal to the junction. Severe vitamin A deficiency, as shown in Fig. 4*F*, causes a near complete replacement of the columnar epithelium of the endocervical canal and glands by stratified keratinized layers of cells. K1 expression is found uniformly on the endocervical canal and glands of extremely deficient mice (Fig. 4*F*). We also observed a marked influence of retinoid deficiency on the expression of K1 in the subjunctional epithelium. This is shown in Fig. 5. Clearly K1 positivity in the ectocervix of mice fed a diet containing 3 μg RA/g of diet becomes manifest four to five cell layers

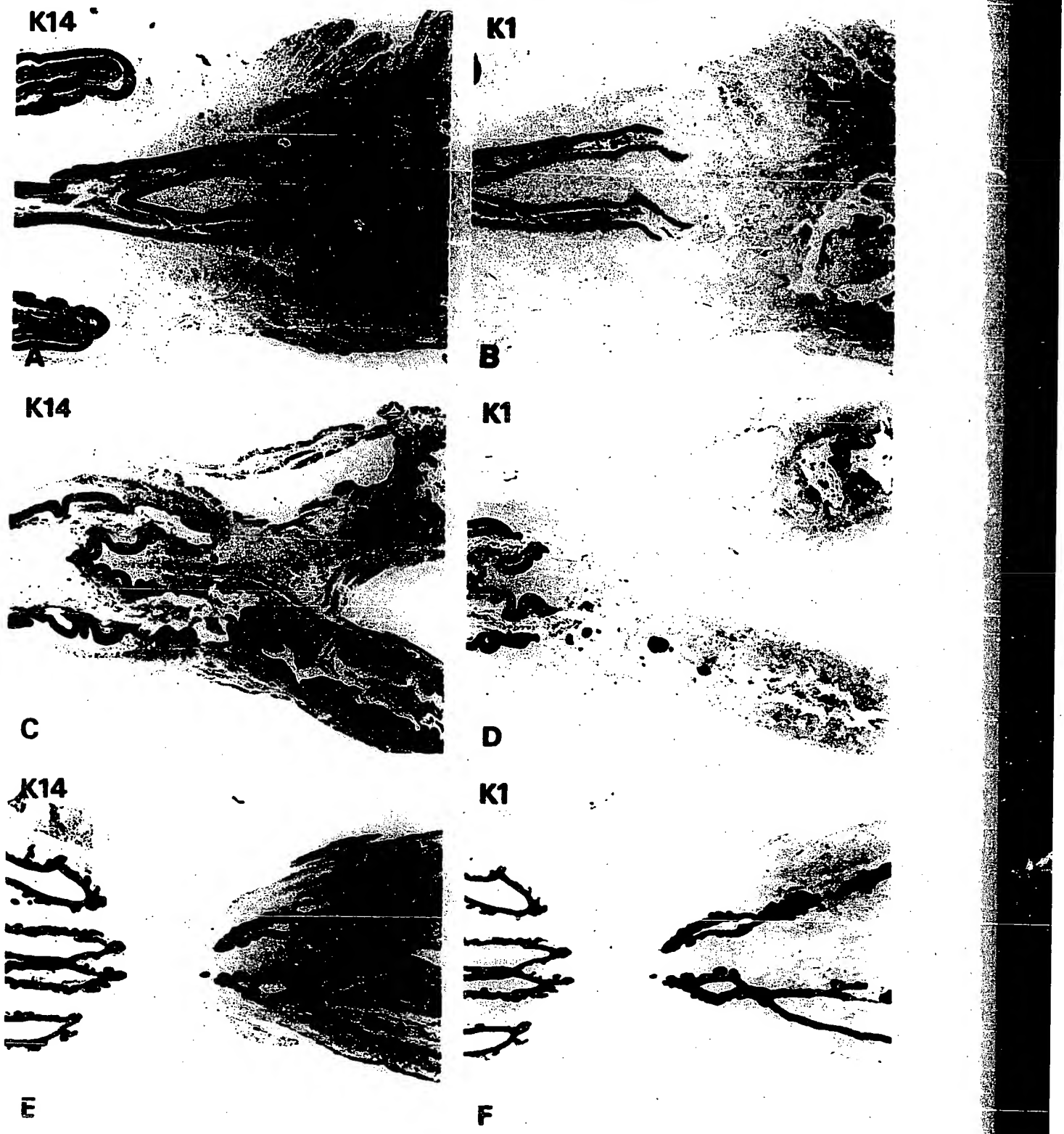

Fig. 4. Immunohistochemistry of keratins K14 (*A, C, E*) and K1 (*B, D, F*) in cervical sections of BALB/c mice. *A, B*, RA+ diet; *C, D*, mild vitamin A deficiency; *E, F*, severe vitamin A deficiency. × 17.

above the basement membrane in areas distal (Fig. 5*A*) or proximal (Fig. 5*B*) to the junction. Moderate or severe vitamin A deficiency causes staining for K1 in all cell layers except for the basal cell layer in areas distal (Fig. 5, *C* and *E*) or proximal (Fig. 5*B*) to the junction, focally for the suprajunctional epithelium in mild deficiency (Fig. 5*D*) and uniformly in severe deficiency (Fig. 5*F*).

Expression of Keratins K6 and K13. Expression of the proliferation marker keratin K6 was most evident subjunctionally in the ectocervical epithelium, with occasional positive cells suprajunctionally (Fig. 6*A*). Mild deficiency caused the appearance of K6-positive foci (Fig. 6*C*) proximal and distal to the junction. K6 expression became uniform in the sub- and suprajunctional epithelium of severely

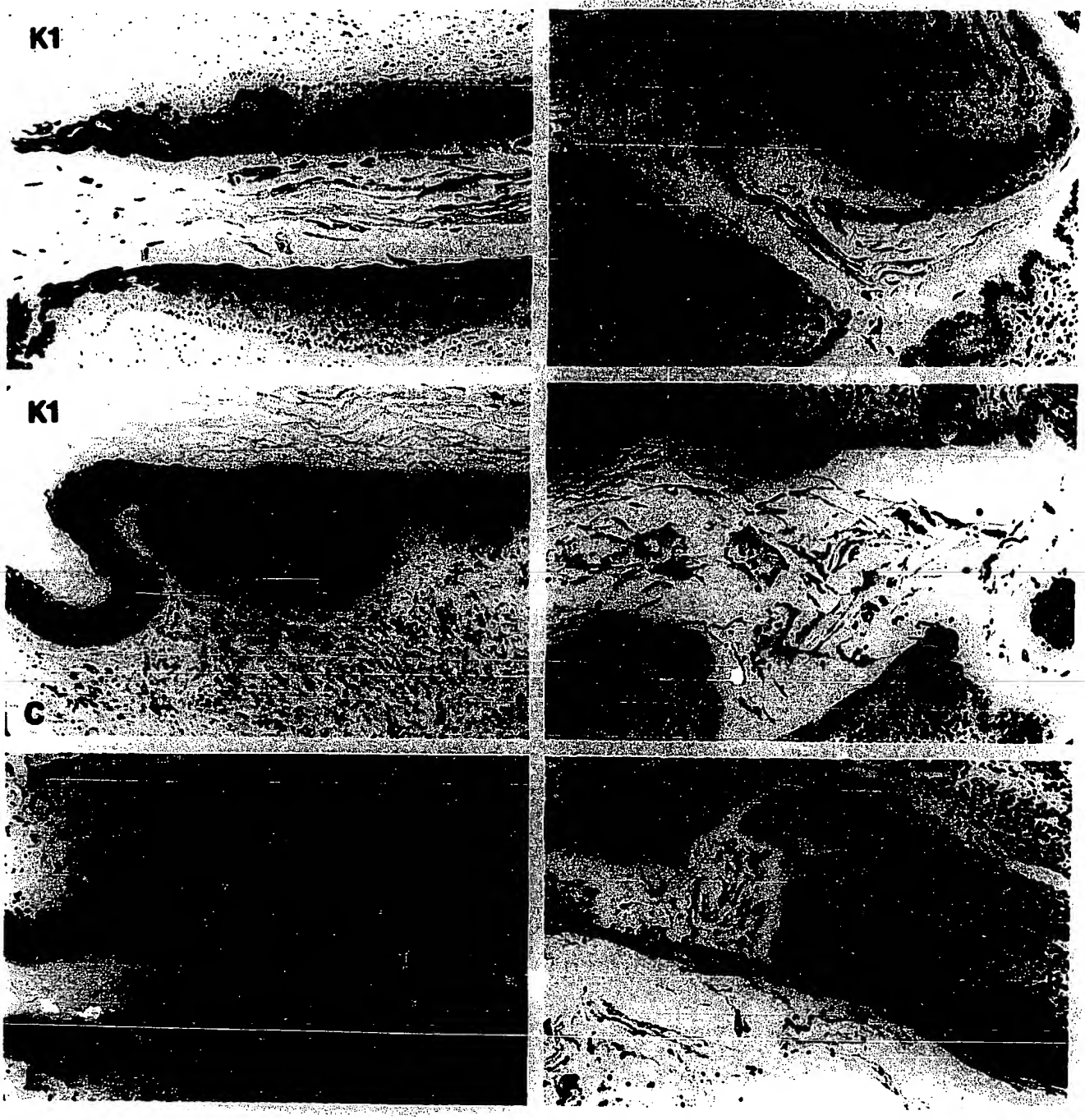

Fig. 5. Localization of keratin K1 in cervical sections of BALB/c mice. *A, B*, RA+ diet; *C, D*, mild vitamin A deficiency; *E, F*, severe vitamin A deficiency. *A, C, E*, subjunctional stratified squamous epithelium; the area comprising the junction is visible in *B* and *D* but not in *F* because of total epithelial replacement with a squamoid stratified epithelium. × 140.

deficient mice (Fig. 6*E*). A unique modulation by retinoid status was observed for the expression of keratin K13, a marker of internal stratified epithelia. Whereas suprajunctional expression increased in vitamin A deficiency, as for most other keratins (Fig. 6, *D* and *F*), an unexpected reduction in K13 expression in the subjunctional ectocervical epithelium was found due to the deficiency (Fig. 6, *B, D*, and *F*). Basal and suprabasal expression of K6 can be observed subjunctionally in the ectocervical epithelium at higher magnification (Fig. 7*A*). In mild deficiency (Fig. 7*C*) the majority of the basal cells fail to express K6; in severe deficiency the basal cells are uniformly negative for K6, both distally (Fig. 7*E*) and proximally to the junction (Fig. 7*D*). In the suprajunctional epithelium, K6-positive cells become evident focally in mild deficiency (Fig. 7*D*) and uniformly in severe deficiency (Fig. 7*F*) in a suprabasal pattern, as for the ectocervix at this stage of deficiency (Fig. 7*F*). Basal and suprabasal localization of K13 was found (Fig. 8*B*) subjunctionally in the ectocervical epithelium proximal to the junction only in mice fed the RA-containing diet. More distally from the junction, ectocervical expression of K13 was always suprabasal (Fig. 8*A*) and it became even more distant from the basal layer with vitamin A deficiency, as clearly shown in Fig. 8, *C* and *E*, for the ectocervix distal from the junction and in Fig. 8*D* for the ectocervical proximal epithelium. Suprajunctional expression of K13 was observed focally at the suprabasal level, both in squamous foci (Fig. 8*D*) and uniformly in squamous epithelium (Fig. 8*F*). Table 1 summarizes all the above data.

Expression of Keratin K5 and K1 mRNA. K5 mRNA was exclusively expressed in the subjunctional epithelium and abruptly stopped at the squamocolumnar junction in cervices from mice fed

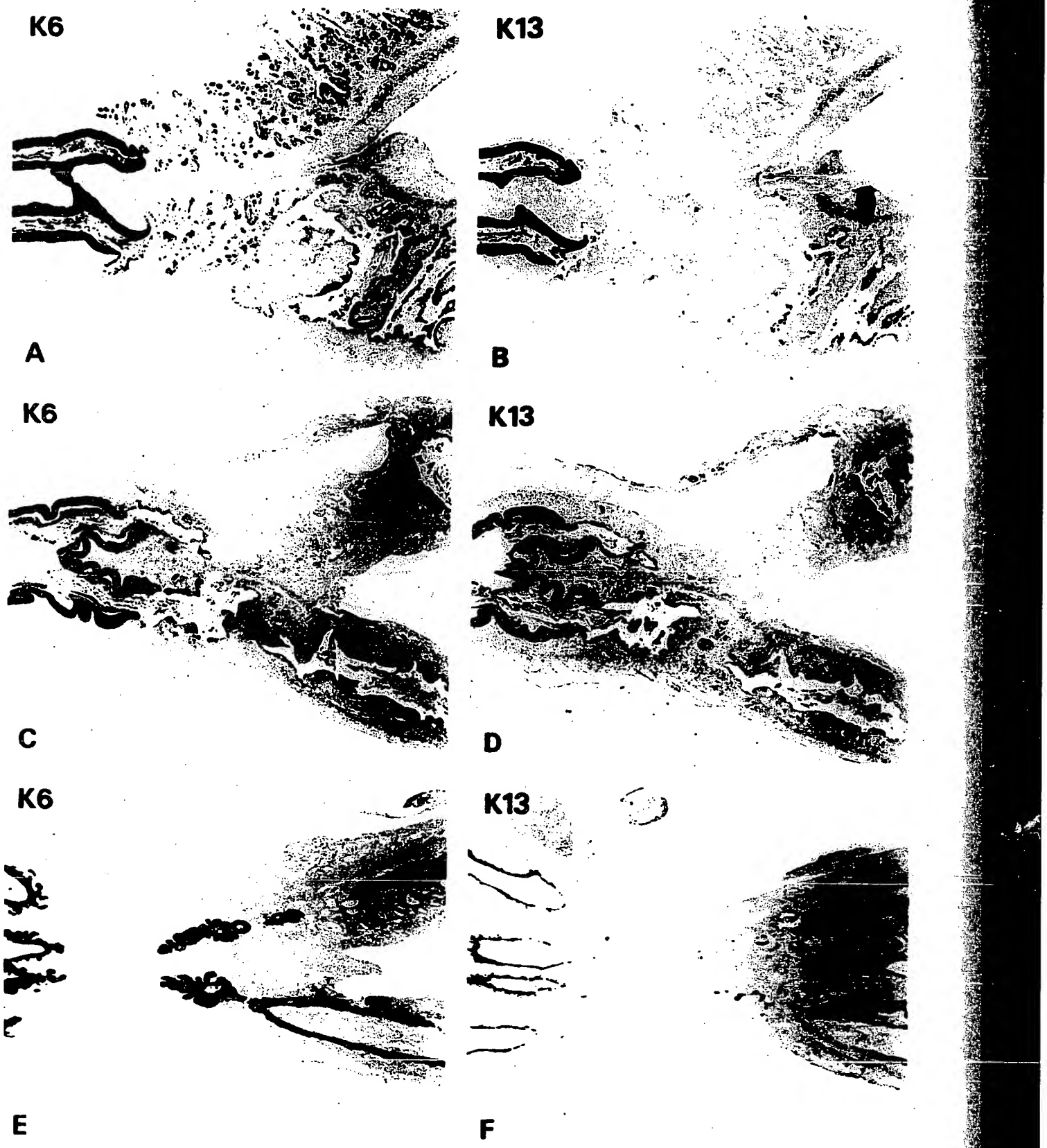

Fig. 6. Immunohistochemistry of keratins K6 (*A, C, E*) and K13 (*B, D, F*) in cervical sections of BALB/c mice. *A, B*, RA+ diet; *C, D*, mild vitamin A deficiency; *E, F*, severe vitamin A deficiency. × 17.

the RA-containing diet (Fig. 9, *A* and *B*). It was mostly associated with the basal cell layer in RA+ (Fig. 9*C*) and RA− subjunctional epithelium (results not shown). K5 mRNA was detected suprajunctionally only under vitamin A deficiency conditions (Fig. 9, *E* and *F*), mostly in the basal cell layers. K1 mRNA was expressed suprabasally in RA+ (Fig. 10, *A* and *B*) and RA− (results not shown)

subjunctional epithelium. It appeared suprajunctionally only under vitamin A deficiency conditions (Fig. 10, *C* and *D*). Similar data were obtained in BALB/c mice (not shown).

DISCUSSION

Retinoids have recently received considerable attention in cancer research because they control phenotypic expression and function of epithelial tissues. In particular, retinoids have been shown to be essential for the maintenance of the mucociliary phenotype in a variety of epithelial linings, which, under conditions of vitamin A deficiency, undergo squamous differentiation and eventually keratinize (17). Steroid hormones induce changes similar to that of vitamin A deficiency in a variety of tissues, including the cervix uteri and vagina. In addition to their effect on mucociliary epithelia, retinoids exert a marked effect on squamous epithelia (36, 37). Application of vitamin A to chick embryonal ectodermal explants has been shown to cause replacement of the epithelium by a mucociliary epithelium (38). Moreover application of RA to human skin causes profound changes in keratin expression (39).

The cervix uteri offers uniquely favorable features, in that it presents with both types of epithelial differentiation; it is stratified squamous, subjunctionally, in the ectocervix and vagina and simple columnar, suprajunctionally, in the endocervical canal and glands under normal conditions. These two phenotypes meet at the squamocolumnar junction as shown in Fig. 1. It is in the area of this junction, or transformation zone, that the neoplastic process usually takes place, following the formation of the preneoplastic lesion, squamous metaplasia (40, 41). The appearance of subcolumnar basal-like cells (re-

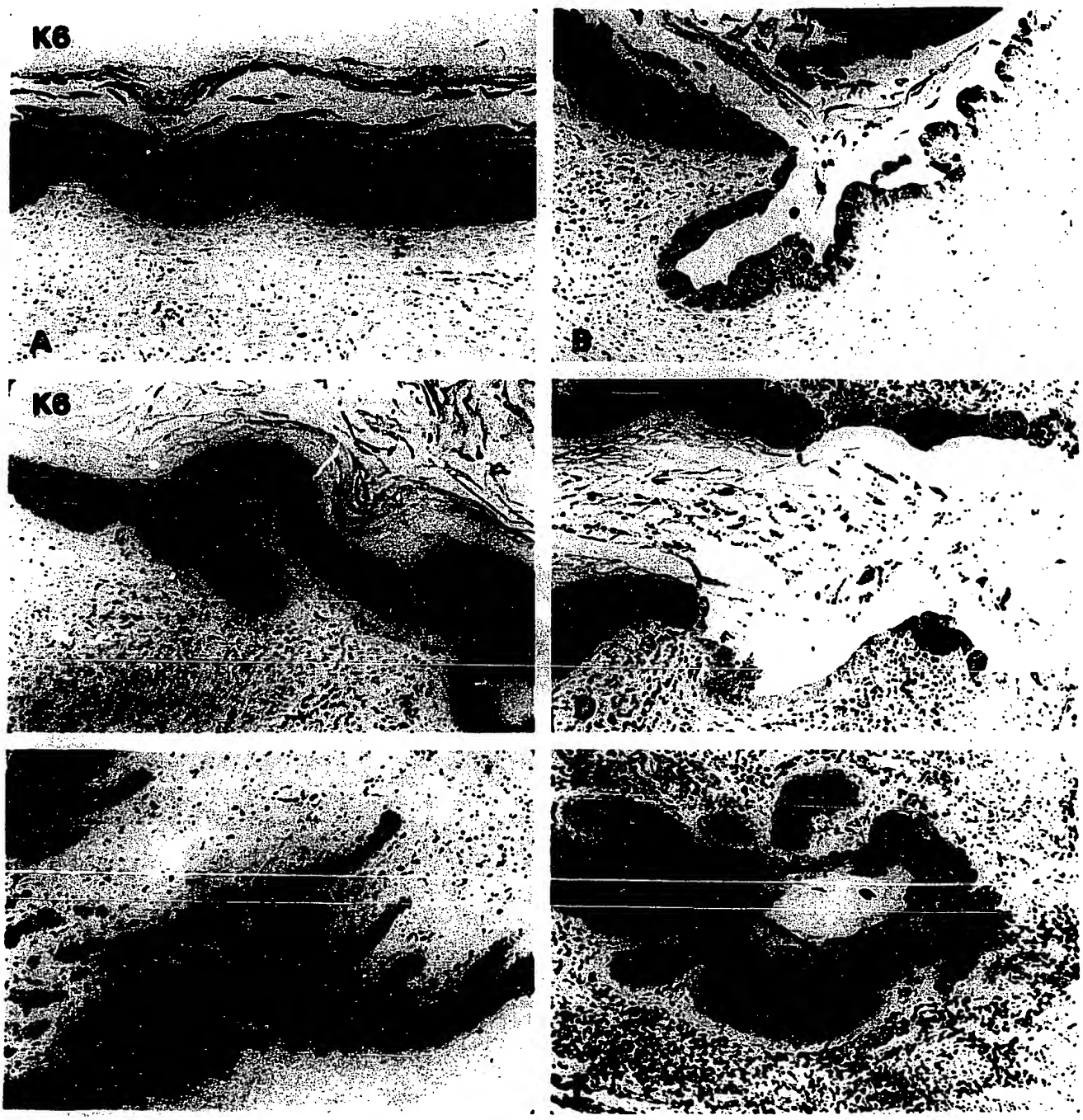

Fig. 7. Localization of keratin K6 in cervical sections of BALB/c mice. *A, B*, RA+ diet; *C, D*, mild vitamin A deficiency; *E, F*, severe vitamin A deficiency. *A, C, E*, subjunctional stratified epithelium; the area comprising the junction is visible in *B* and *D* but not in *F* because of total epithelial replacement with a squamoid stratified epithelium. × 140.

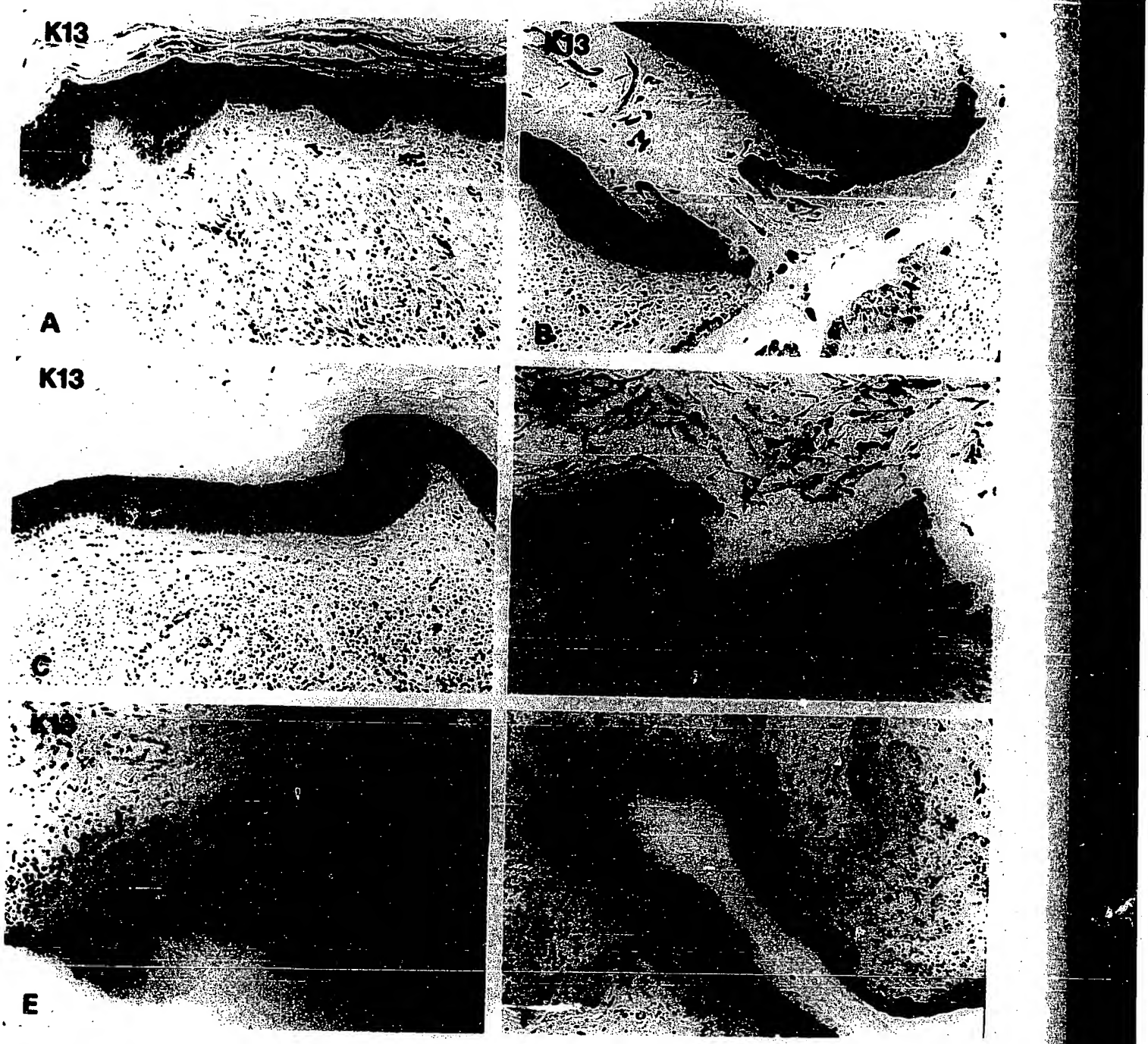

Fig. 8. Localization of keratin K13 in cervical sections of BALB/c mice. *A, B,* RA+ diet; *C, D,* mild vitamin A deficiency; *E, F,* severe vitamin A deficiency. *A, C, E,* subjunctional epithelium; the area comprising the junction is visible in *B* and *D* but not in *F* because of total epithelial replacement with a squamoid stratified epithelium. × 140.

Table 1 *Keratin expression and localization in cervical sections of normal and vitamin A-deficient mice*[a]

	RA+ diet		Mild deficiency		Severe deficiency	
	Suprajunctional	Subjunctional	Suprajunctional	Subjunctional	Suprajunctional	Subjunctional
K5, K14	+++ Basal, supra-basal	+++ Basal, supra-basal	+ Basal, supra-basal	+++ Basal, supra-basal	+++ Basal, supra-basal	+++ Basal, supra-basal
K8	+++	–	++	–	+	–
K1	–	++ Suprabasal	+ Suprabasal	+++ Suprabasal	+++ Suprabasal	+++ Suprabasal
K6	+	+++ Basal, supra-basal	+ Mainly supra-basal	++ Mainly supra-basal	++ Suprabasal	+++ Suprabasal
K13	–	+++ Basal, supra-basal	+ Suprabasal	++ Suprabasal	+ Very suprabasal	+ Very suprabasal

[a] The intensity of expression of the different keratins is indicated relative to the expression in normal cervix. –, absent; +, focal staining (less than 5% of epithelium); ++, moderate staining (less than 40% of epithelium); +++, extensive distribution (90 to 100% of epithelium).

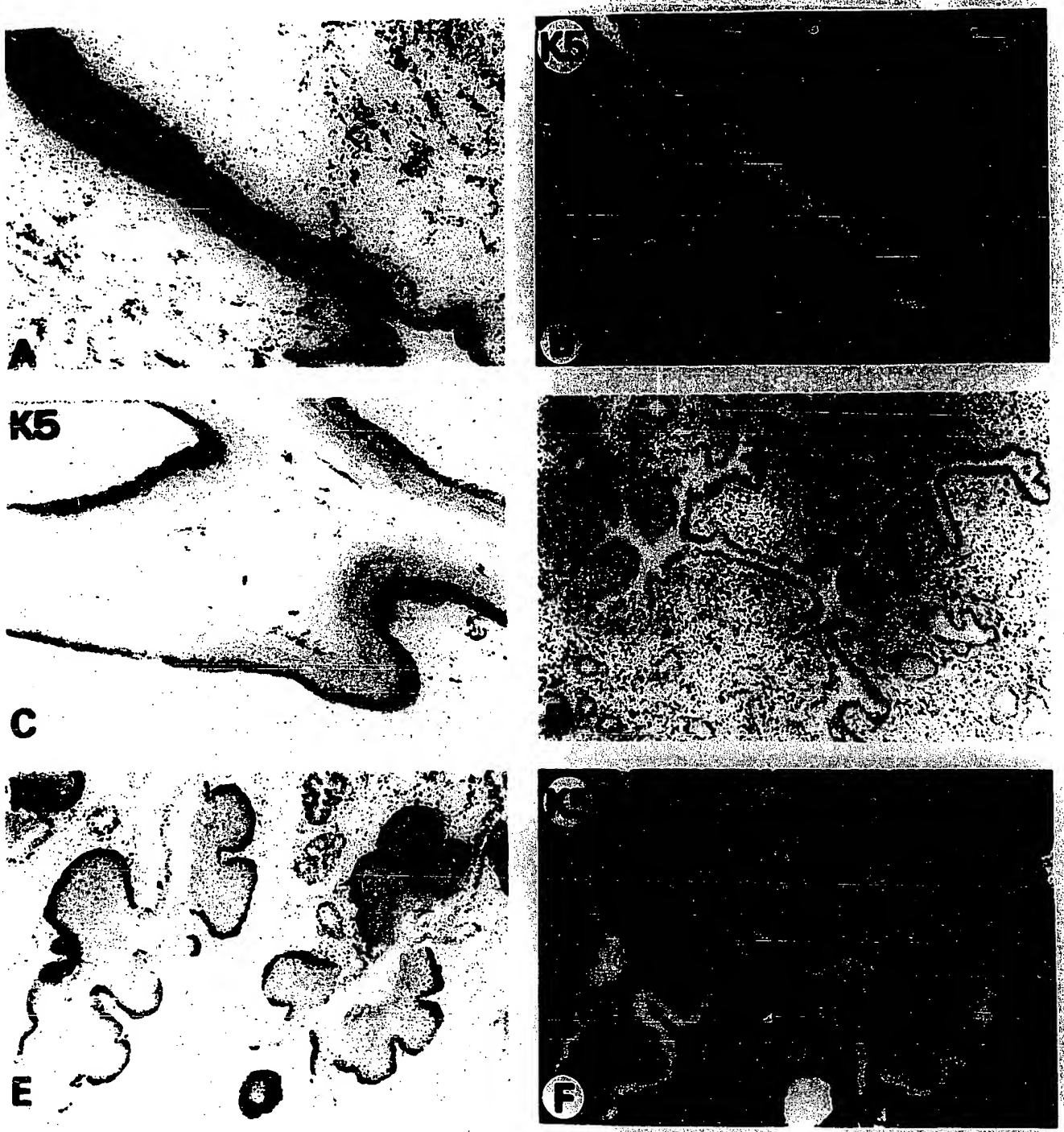

Fig. 9. *In situ* hybridization of K5 mRNA in cervical sections of nude mice. *A, B, C, D,* RA+ diet; *E, F,* severe vitamin A deficiency. *A, B,* at squamo-columnar junction; *C,* subjunctional epithelium; *D, E, F,* suprajunctional glandular epithelium. × 70.

serve cells), not normally present in the suprajunctional epithelium, precedes squamous metaplasia and neoplasia (42). Therefore these cells have been implicated in the process of malignant transformation. In previous studies, the various keratins expressed in normal and pathological specimens of the human female genital tract have been identified (21, 43–46). A recent study has demonstrated the presence of basal cell keratins K5, K14, and K17 in cervical reserve cells (47). It was also shown that a considerable number of premalignant lesions of the uterine cervix express the same keratins as found in the progenitor reserve cells (48). During progression of cervical intraepithelial neoplasia an increase in keratin 17 was observed (47).

This murine model permits the development of endocervical and endometrial squamous lesions by nutritional deprivation of vitamin A and is particularly useful to study the histogenesis of squamous metaplasia and observe the emergence of subcolumnar reserve cells, the precursors of the squamous lesions, at sporadic sites proximal and distal from the squamo-columnar junction. It also permits the reversal of the lesion by nutritional repletion with RA. In our nutritionally controlled mice the presence of RA in the diet did not permit the appearance of these reserve cells. In retinoid-deprived mice of different strains (BALB/c and nude), however, reserve cells could be observed by immunohistochemical staining and *in situ* hybridization with specific probes to keratin K5. These cells were observed proximal and distal from the junction, as isolated cells or as a contiguous row (results not shown) of single cells in subcolumnar position. By *in situ* hybridization we identified single subcolumnar reserve cells ex-

Fig. 10. *In situ* hybridization of K1 mRNA in cervical sections of nude mice. *A, B,* RA + diet; *C, D,* severe vitamin A deficiency. *A,* subjunctional epithelium; *B, C, D,* suprajunctional glandular epithelium. × 70.

pressing K5 mRNA underneath the columnar epithelium. Isolated reserve cells could not usually be observed using antibodies to K6, K1, K10, or K13. Fully developed squamous foci, several cell layers thick, were uniformly positive for K5 and K14 but showed only suprabasal staining for K6, K1, K10, and K13, indicating that undifferentiated reserve cells occupy a basal position in these foci. Squamous foci and their precursor reserve cells were uniformly negative for keratin K8, which was found expressed in columnar cells of the normal simple suprajunctional epithelium, as expected from the work of Moll *et al.* (21) and Franke *et al.* (43). In severe vitamin A deficiency, K8 staining was prominently absent, inasmuch as the near totality of the epithelium was replaced by squamous keratinizing cells spanning the length of the cervix (ecto- and endo-) and glands.

Keratin production in cultured human endocervical cells has been studied by Turyk *et al.* (49), who reported the presence of keratin K13 in late passage endocervical cells, even though this keratin was not detected in early passage cells. We suggest, in the light of our data, that the absence of serum in their culture medium may have caused the induction of K13 synthesis as a result of squamous differentiation caused by vitamin A deficiency, just as we find *in vivo*, in the endocervical epithelium.

Our studies also show that the ectocervical epithelium becomes keratinized, due to vitamin A depletion in a manner similar to the induction of keratinization by estrogen. Similar changes are clearly observable in the estrous phase of the menstrual cycle in the ectocervical vaginal epithelium. We found that expression of K5 and K14 is uniform in basal and suprabasal cells, but expression of K6 and K13 is uniform in all epithelial cell layers throughout the ectocervix, with only intense staining in basal cells near the junction for both keratins. In vitamin A deficiency, K6 and K13 are expressed mainly in suprabasal cells but with a difference; whereas K6 expression became negative in the basal cell layer but was retained in all other layers, K13 expression was detected only several cell layers above the basement membrane.

Previous work has shown that cultured ectocervical epithelial cells behave like ectocervical cells *in vivo*, in that they stratify, produce envelopes, and express keratins K5, K6, K13, K14, K16, K17, and K19 (16, 50). These human cells failed to express K1 in culture. Steroid hormone greatly enhanced and natural and synthetic retinoids greatly reduced the cornification of these cells (16).

Our studies show that vitamin A deficiency had no effect on the distribution of K1 in the different cell layers but that it increased the intensity of K1 expression in the vitamin A-deficient ectocervix. K10 expression followed K1. *In situ* hybridization studies for K1 and K5 mRNA showed their major site of expression at the basal (K5) and immediately suprabasal (K1) cell layers. The localization of both K5 and K1 proteins in these same cell layers and above is consistent with transcriptional regulation of these keratins (23). It is therefore obvious from this work and the work of other investigators that retinoid status exerts a profound influence on the differentiation of cervical epithelial cells.

The differentiation of cervical epithelium may yield some useful insight into our understanding of mechanisms responsible for the establishment of cervical neoplasia. Cervical cancer is a major public health problem worldwide (51).

HPV infection has been linked to cervical cancer and the presence of specific viral oncoproteins to the expression of the malignant phe-

notype of HPV-positive cervical cancers (for a review see Ref. 52). Moreover HPV replication has been shown to be associated with the differentiating layers of the cervical epithelium (53) and foreskin. The transcription of HPV genes (54) and the transformation of human keratinocytes by HPV-16 (55) were found to be inhibited by RA. RA was also shown to decrease DNA copy number for bovine papilloma virus type 1 (56). Moreover Agarwal *et al.* (57) have suggested that retinoids may reduce the extent of HPV-16 infection and thus may slow down the neoplastic process by inhibiting squamous differentiation of ectocervical epithelial cells.

Several epidemiological studies have demonstrated an inverse correlation between dietary intake or blood levels of vitamin A, retinol-binding protein and/or carotenoids and cancer risk at several epithelial sites (58–61), including the cervix (61–65). Moreover several studies suggest that retinoids might be effective in reversing and treating premalignant lesions such as cervical intraepithelial neoplasia (66–71). Phase I and phase II clinical trials of RA for cervical intraepithelial neoplasia have shown that RA can reverse cervical dysplasia in some patients (66–71). In addition, Pirisi *et al.* (54) have shown that human foreskin keratinocyte cell lines immortalized by transfection with HPV16 DNA are more sensitive than normal human keratinocytes to growth control and modulation of keratin expression by RA and retinol. These authors also found that RA reduces by 2- to 4-fold the expression of the viral proteins E6 and E7 in a dose- and time-dependent manner.

These data represent promising leads into possible preventive and therapeutic strategies of cervical neoplasia by retinoids. The interaction among steroid hormones, retinoids, and their receptors in maintaining the typical differentiation characteristics of the cervical epithelia is of considerable interest and represents the focus of our investigation.

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